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Selective inhibition of extra-synaptic $\alpha 5$ -GABA_A receptors by S44819, a new therapeutic agent

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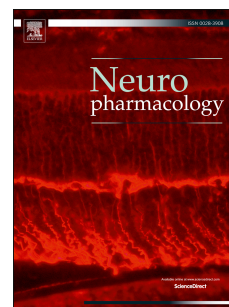
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Abbreviations

$\alpha 5$ -GABA_ARs = $\alpha 5$ subunit containing GABA_A receptors

BW= body weight

CI = Confidence interval

CNS = central nervous system

eIPSC = electrically evoked inhibitory postsynaptic current

fEPSP = field excitatory postsynaptic potential.

GABA = Gamma-aminobutyric acid

GABA_ARs. = GABA_A receptors

LTP = long term potentiation

mIPSC = miniature inhibitory postsynaptic current

NAM = Negative allosteric modulator.

sIPSC = spontaneous inhibitory postsynaptic current

TBS = theta burst stimulus.

TE = total number of errors

TTX = tetrodotoxin

V_h = holding potential

WT = wild type

Abstract

In the mammalian central nervous system (CNS) GABA_A receptors (GABA_ARs) mediate neuronal inhibition and are important therapeutic targets. GABA_ARs are composed of 5 subunits, drawn from 19 proteins, underpinning expression of 20-30 GABA_AR subtypes. In the CNS these isoforms are heterogeneously expressed and exhibit distinct physiological and pharmacological properties. We report the discovery of S44819, a novel tricyclic oxazolo-2,3-benzodiazepine-derivative, that selectively inhibits α 5-subunit-containing GABA_ARs (α 5-GABA_ARs). Current α 5-GABA_AR inhibitors bind to the “benzodiazepine site”. However, in HEK293 cells expressing recombinant α 5-GABA_ARs, S44819 had no effect on ³H-flumazenil binding, but displaced the GABA_AR agonist ³H-muscimol and competitively inhibited the GABA-induced responses. Importantly, we reveal that the α 5-subunit selectivity is uniquely governed by amino acid residues within the α -subunit F-loop, a region associated with GABA binding. In mouse hippocampal CA1 neurons, S44819 enhanced long-term potentiation (LTP), blocked a tonic current mediated by extrasynaptic α 5-GABA_ARs, but had no effect on synaptic GABA_ARs. In mouse thalamic neurons, S44819 had no effect on the tonic current mediated by δ -GABA_ARs, or on synaptic (α 1 β 2 γ 2) GABA_ARs. In rats, S44819 enhanced object recognition memory and reversed scopolamine-induced impairment of working memory in the eight-arm radial maze. In conclusion, S44819 is a first in class compound that uniquely acts as a potent, competitive, selective antagonist of recombinant and native α 5-GABA_ARs. Consequently, S44819 enhances hippocampal synaptic plasticity and exhibits pro-cognitive efficacy. Given this profile, S44819 may improve cognitive function in neurodegenerative disorders and facilitate post-stroke recovery.

Key Words:

Tonic inhibition; phasic inhibition; α 5-GABA_A receptors; extrasynaptic GABA_A receptors; long term potentiation; cognition.

Highlights:

All current, selective, inhibitors of $\alpha 5$ -GABA_ARs bind to the “benzodiazepine site”.

S44819 acts *via* a unique site on the $\alpha 5$ -GABA_AR to competitively inhibit GABA binding.

S44819 inhibits mouse hippocampal tonic, but not phasic inhibition & facilitates LTP.

In rats S44819 enhanced novel object recognition & spatial working memory.

This novel $\alpha 5$ -GABA_AR site offers a new target to treat cognitive disorders & stroke.

S44819 has successfully completed a phase1 clinical trial.

1. INTRODUCTION

1.1) *GABA_A receptors.* Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the mammalian central nervous system (CNS), that influences neuronal activity by activating ionotropic anion-conducting GABA_A receptors (GABA_ARs) and metabotropic GABA_B receptors (Olsen and Sieghart, 2008; Rudolph and Möhler, 2014). GABA_ARs are members of the Cys-loop ligand-gated ion-channel family, which are composed of five subunits, assembled from a repertoire of 19 proteins (Olsen and Sieghart, 2008). This diversity underpins the expression of 20-30 GABA_AR subtypes, which exhibit distinct physiological and pharmacological properties (Olsen and Sieghart, 2008; Rudolph and Möhler, 2014). The topographical CNS distribution of GABA_AR subunits suggests non-redundant functions (Fritschy and Panzanelli, 2014; Pirker et al., 2000; Wisden et al., 1992) and by inference, drugs that exhibit GABA_AR isoform selective actions may present a behavioural profile distinct from that of non-selective agents. In support, mice genetically engineered to express benzodiazepine-insensitive α -subunits revealed that the diverse behavioural effects of diazepam are attributed to receptors incorporating particular α -subunits (Rudolph and Möhler, 2014).

1.2) *α 5-GABA_A receptors: physiology & pharmacology.* Of interest here are GABA_ARs incorporating the α 5 subunit (α 5-GABA_ARs). In humans, PET imaging studies with Ro15-4513 suggest α 5-GABA_ARs to be expressed in hippocampus, cortex and ventral striatum (Mendez et al., 2013). In rodents this receptor is expressed in cortex, amygdala, olfactory bulb and spinal cord, but is particularly densely expressed in the hippocampus (Rudolph and Mohler, 2014). In the rodent hippocampus, α 5-GABA_AR are located primarily, but not exclusively, extra-synaptically, where in CA1 pyramidal neurons they mediate tonic inhibition and contribute to occasional slow inhibitory phasic events (Capogna and Pearce, 2011; Caraiscos et al., 2004; Prenosil et al., 2006; Zarnowska et al., 2009). Mice lacking the α 5 subunit exhibit an enhanced performance in various cognitive tasks (Atack, 2011; Martin et al., 2009; Rudolph and Möhler, 2014), although present with an impairment of executive

function (assessed by a puzzle box test) and short-term memory (Zurek et al., 2016). In rodents, selective benzodiazepine-site $\alpha 5$ -GABA_AR negative allosteric modulators (NAMs) reduce the CA1 tonic current and display pro-cognitive activity in certain behavioural paradigms (Atack, 2011). The expression of hippocampal $\alpha 5$ -GABA_ARs is dynamic *e.g.* being altered by inflammatory cytokines and in mouse models of Alzheimer's disease, a plasticity associated with impaired memory performance (Wang et al., 2012; Wu et al., 2014). Due to their therapeutic potential in treating moderate cognitive impairment (Atack, 2011) and improving recovery from stroke (Clarkson et al., 2010), there is considerable interest in developing drugs that selectively inhibit $\alpha 5$ -GABA_AR function (Atack, 2011; Bolognani et al., 2015; Darmani et al., 2016).

All current selective $\alpha 5$ -GABA_AR inhibitors bind to the benzodiazepine recognition site, located between the $\alpha 5$ and $\gamma 2$ subunit interface, where they act as NAMs, to reduce GABA efficacy (Rudolph and Möhler, 2014). We reported the synthesis and partial biological characterization of a family of tricyclic compounds based on an oxazolo-2,3-benzodiazepine scaffold (Ling et al., 2012; Ling et al., 2015). In contrast to classical 1,4-benzodiazepines, *e.g.* diazepam and to current $\alpha 5$ -GABA_AR NAMs, these compounds are orthosteric inhibitors of GABA binding, acting at the α - β subunit interface. For some compounds this property is paired with inhibition of channel gating, similar to the properties of the GABA_AR antagonist bicuculline (Ueno et al., 1997). Here, we demonstrate that a new member of this series, S44819, is potentially the most valuable. S44819 is an orthosteric, competitive, $\alpha 5$ -GABA_AR-selective antagonist. Distinct from known $\alpha 5$ -GABA_AR NAMs, the α -subunit specificity is governed by amino acid residues located in the $\alpha 5$ -subunit F-loop, a region associated with GABA binding. In contrast to bicuculline, S44819 has no effect on the activation of $\alpha 5$ -GABA_ARs by etomidate. In CA1 pyramidal neurons, S44819 selectively blocked the tonic current mediated by extrasynaptic $\alpha 5$ -GABA_ARs, with no effect on the phasic currents mediated by synaptic GABA_ARs and consequently enhanced submaximal long-term potentiation (LTP). Further emphasising selectivity, in ventrobasal (VB) thalamic

neurons, S44819 had no effect on the tonic or phasic conductance mediated by extrasynaptic δ -GABA_ARs and synaptic $\alpha 1\beta 2\gamma 2$ -GABA_ARs (Belelli et al., 2005).

Although acting on the receptor in a manner distinct from established $\alpha 5$ -GABA_AR NAMs, in common, in rats S44819 enhanced object recognition memory and reversed scopolamine-induced impairment of spatial working memory in the eight-arm radial maze. Therefore, S44819 provides a new mechanistic tool to elucidate the role of $\alpha 5$ -GABA_ARs in neuronal signalling and behaviour. Given this profile S44819 has recently undergone a successful phase 1 clinical trial (Darmani et al., 2016).

2. MATERIALS AND METHODS

2.1) Compounds.

S44189 (Egis-13529), a substituted *8-methyl-5-[1-benzothiophen-2-yl]-1,9-dihydro-2H-[1,3]oxazolo[4,5-h][2,3]benzodiazepin-2-one*, was produced at greater than 97% purity at Egis Pharmaceuticals PLC as previously described (Ling et al., 2012; Ling et al., 2015). All chemical and biochemical reagents were of the highest grade available. For all *in vitro* experiments a stock solution of S44819 (10 mM) was dissolved in 100% DMSO aliquoted and stored at -20 °C. Note that for the *in vitro* assays 0.1 % DMSO had no effect on the various control recordings made. For all *in vivo* studies a nano-encapsulated preparation of S44819 was used. The S44819 (1% w/w) was dispersed with Lipoid S100 (10% w/w Lipoïd GmbH, Ludwigshafen, Germany) and Trehalose (15% w/w) in water. The suspension was stirred for 3h at 50 °C and homogenized with an Ultraturax® T25 digital at 20,000 rpm during 2 min 30 s. The milling of the suspension was then performed with 50ml of zirconium oxide beads at 5,000 rpm during 90 min with a DynoMill RL equipment. The particle size was measured during the milling by laser diffraction and/or dynamic light scattering (DLS). At the end of the process a filtration of the suspension was performed with 25 µm filters. The particle size obtained was $D(50, v) = 130$ nm. In order to prevent crystal growth and

particle agglomeration, the suspension was then freeze-dried. Control experiments used the vehicle (Lipoïd S100/trehalose, 40%/60%, w/w).

2.2) Cell culture.

HEK293 cell lines, stably expressing recombinant GABA_ARs were obtained upon transfection with the cDNAs encoding the human α -subunit isoforms, together with the cDNAs for rat β_2 (short) and rat γ_2 (long) in antibiotic resistance variants of the expression vector pcDNA3.1 (Life Technologies, Inc., Carlsbad, CA, USA), or pExchange (Agilent Technologies, Inc., Santa Clara, CA, USA) as previously described (Ling et al., 2015). The GABA_AR $\alpha\beta_2\gamma_2$ cell lines were maintained under triple antibiotic selection with neomycin (0.6 mg/ml), zeocin (0.2 mg/ml) and puromycin (3 μ g/ml) in Dulbecco's DMEM supplemented with 10 v/v% foetal bovine serum and 10 mM KCl. Further cell lines, one co-expressing GABA_AR β_2 - and γ_2 -subunits, another expressing the γ_2 -subunit were propagated under selection with neomycin (0.6 mg/ml) and zeocin (0.2 mg/ml), or zeocin (0.2 mg/ml), respectively, in Dulbecco's DMEM supplemented with 10 v/v% foetal bovine serum.

Transient transfections were made with the calcium-phosphate method (Salmon and Trono, 2006) utilising the desired expression vectors of the requisite wild-type (WT) and variously mutated α -subunit cDNAs for functional studies. The cells were collected 48 h later, aliquoted and frozen in foetal bovine serum supplemented with 10% (v/v) DMSO and stored at -150°C until further use.

2.3) Site-directed mutagenesis.

Mutations were introduced by site-directed mutagenesis with custom-designed primers using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific Waltham, MA USA) as *per* the manufacturer's instructions. The resultant constructs were verified by DNA sequencing of the entire coding region (Biomi Ltd, Gödöllő, Hungary).

2.4) FLIPR dye assay.

For fluorescence recordings, cells were plated in black, clear-bottom, half-volume 96-well plates coated with poly-*D*-lysine at 50,000 cells *per* well in 50 μ l of growth medium containing neomycin, zeocin and puromycin and used 24 h later. Briefly, the cells were washed and incubated at 37°C in 20 mM HEPES buffered Hank's Balanced Salt Solution (HBSS), pH 7.4 supplemented with FLIPR Membrane Potential Assay Kit BLUE (Molecular Devices) dye at 2x dilution. The dye solution contains a voltage-sensitive fluorescent dye and an extracellular fluorescence quench-substance. Simultaneously with dye-loading, various concentrations of the test compound dissolved in HBSS supplemented with 20 mM HEPES, pH 7.4 and 1% (v/v) DMSO were added to the incubation medium at 37 °C. The final concentration of DMSO in the medium was 0.1% (v/v). Forty minutes later the plates were placed into a Flexstation3 (Molecular Devices) plate reader and the recording of fluorescence commenced — designated as time 0. Subsequently, the fluorescence signal was sampled at 2 s intervals, the stimulus (GABA, or etomidate) was introduced at 30 s and the recording was terminated at 120 s after time 0. The area under the curve of time vs. relative fluorescence units after the addition of agonist was calculated with the average baseline subtracted by Soft-Max 5.4 software and used for further analysis. Non-linear regression curves were fitted using GraphPad Prism version 6.0 and the EC₅₀, or IC₅₀ values were derived from fit to the curve. If otherwise not indicated, to make the data comparable between experiments, the results are expressed as a percentage of the response evoked by 1.6 μ M GABA.

2.5) Radioligand-binding studies.

HEK-293T (ACC 635) cells were transfected with the calcium-phosphate co-precipitation method (Salmon and Trono, 2006) with cDNAs encoding the GABA_AR human α 5, human β 3, rat γ 2 (long) subunits in the expression vector pcDNA3.1 (Life Technologies, Inc., Carlsbad, CA, USA) and pExchange (Agilent Technologies, Inc., Santa Clara, CA, USA)

(Ling et al., 2015). The $\beta 3$ subunit was used in binding assays of $\alpha 5$ subunits, as GABA_A receptor binding was much greater than with $\beta 2$ subunits — see also (Lüddens et al., 1994). Cells were harvested and frozen 72 h later. All other procedures assessing the binding of ^3H -muscimol and ^3H -flumazenil to crude membranes prepared from the transfected cells were as previously reported (Ling et al., 2015).

2.6) Whole-cell voltage-clamp studies of recombinant GABA_ARs expressed in HEK 293 cells.

Patch electrodes were filled with a solution comprising (in mM) 140 CsCl, 0.1 CaCl₂, 1.1 EGTA, 10 HEPES, pH 7.2 (adjusted with 1 M CsOH). Whole-cell currents ($V_h = -60$ mV) evoked by the local brief pressure-application (General Valve Picospritzer II) of GABA (100 μM ; one application of 50 ms duration *per min*) were recorded in an extracellular solution consisting of (in mM) 142.8 NaCl, 0.1 MgCl₂, 0.1 CaCl₂, 10 glucose, 10 HEPES, pH 7.2 (adjusted by 1 M NaOH). Three control GABA-evoked currents of amplitudes within 10% of each other were obtained before perfusing S44189 from a reservoir into the recording chamber.

2.7) Preparation of mouse hippocampal and thalamic brain slices for whole-cell voltage-clamp studies.

Hippocampal, or thalamic brain slices were prepared from mice of either sex (p 17 – 24) as we previously described (Brown et al., 2016; Herd et al., 2008). Animals were killed by cervical dislocation in accordance with Schedule 1 of the UK Animals (Scientific Procedures) Act 1986. The brain was rapidly dissected and placed in 'ice-cold', oxygenated artificial cerebrospinal fluid (aCSF-1) solution (in mM): 225 - 234 sucrose, 2.95 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 0.5 CaCl₂, 10 MgSO₄, 10 d-glucose, (pH 7.4; 330 – 340 mosmol l⁻¹). Hippocampal coronal slices (300 μm thick) were cut using a Vibratome (Leica VT1000), transferred onto a nylon mesh platform housed within a chamber containing an oxygenated aCSF-2 composed of (in mM): 126 NaCl, 2.5 KCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 0.5 CaCl₂, 10 d-glucose, 10 MgSO₄, 1 ascorbic acid and 3 Na pyruvate

(pH 7.4; 300–310 mosmol l^{-1} ; room temperature), where they were maintained for a minimum of 1 h prior to experimentation. For thalamic slices, the brain was again maintained in the oxygenated aCSF-1 solution and then sectioned in the horizontal plane using a Vibratome series 1000 PLUS Sectioning System (Intracell, Royston, Hertfordshire, UK). Tissue slices were cut at 300 - 350 μm thickness and then transferred to a chamber containing oxygenated aCSF-3 solution (in mM: 126 NaCl, 2.95 KCl, 26 NaHCO_3 , 1.25 NaH_2PO_4 , 2 CaCl_2 , 2 MgCl_2 , 10 glucose (pH 7.4; 306–309 mosmol l^{-1} ; room temperature) for a minimum of 1 h before recording.

2.8) Whole-cell voltage-clamp recordings:

The extracellular recording solution (ECS) contained (in mM): 126 NaCl, 2.95 KCl, 26 NaHCO_3 , 1.25 NaH_2PO_4 , 2 CaCl_2 , 10 d-glucose, 2 MgCl_2 , (pH 7.4; 300–310 mosmol l^{-1}). Recording electrodes were constructed from thick-walled borosilicate glass (Garner Glass Co., Claremont, CA, USA), using a PC-10 electrode puller (Narashige, Japan) and had open tip resistances of 3 – 6 $\text{M}\Omega$ when containing either intracellular solution-1 (ICS-1) for CA1 neurons comprising (in mM): 135 CsCl, 10 Hepes, 10 EGTA, 2 Mg-ATP, 1 CaCl_2 , 1 MgCl_2 , 5 QX-314 (pH 7.3 with CsOH, 300–305 mosmol l^{-1}), or ICS-2 for thalamocortical neurons (in mM): 140 CsCl, 10 Hepes, 10 EGTA, 2 Mg-ATP, 1 CaCl_2 , 2 MgCl_2 , 5 QX-314 (pH 7.2 – 7.3 with CsOH, 290–300 mosmol l^{-1}). For CA1 recordings the hippocampal slices were incubated in vigabatrin (50 μM ; Sigma Aldrich) for at least 1 h prior to recording to raise the ambient extracellular concentration of GABA (Caraiscos et al., 2004).

Recordings were made at 35°C from hippocampal CA1 pyramidal neurons, or thalamic ventrobasal (VB) neurons visually identified with an Olympus BX51 (Olympus, Southall, UK) microscope equipped with differential interference/infrared optics and a CCD camera. Whole-cell voltage-clamp recordings were acquired at a holding potential (V_h) of -60 mV using an Axopatch 1D amplifier (Molecular Devices, Sunnyvale, CA, USA). Data acquisition and digitization (10 kHz) was performed using a NIDAQmx interface (National

Instruments, Austin TX, U.S.A.). For both VB and CA1 neurons the mIPSCs were isolated by supplementing the ECS with 2 mM kynurenic acid and 0.5 μ M tetrodotoxin (TTX). The mIPSC amplitude was determined as previously described (Brown *et al.*, 2015). A putative effect of S44819 on the mIPSC amplitude was assessed in paired recordings using the paired t test. The tonic current was calculated by determining the outward current produced by bath applied bicuculline methobromide (30 μ M) – (Brown *et al.*, 2015). The effect of S44819 (10 μ M) on the holding current was similarly determined. In quantifying the holding current the mean DC current of a 30 – 60 s current section was determined from sequential 25.6 – 102.4 ms epochs, depending on the mIPSC frequency. All epochs containing mIPSCs, or sections of recordings that were unstable, were not included in the determination of the mean holding current (Brown *et al.*, 2015).

2.9) Preparation of mouse hippocampal brain slices for extracellular recording.

The hippocampal brain slices were obtained from 2 to 4 month old male C57/Bl6 mice and prepared as we previously described (Zhang *et al.*, 2013). The mice were killed by cervical dislocation in accordance with Schedule 1 of the UK Government Animals (Scientific Procedures) Act 1986. After decapitation, the brain was dissected, before being incubated in a chamber containing artificial cerebrospinal fluid (aCSF) composed of (in mM): NaCl 124; NaHCO₃ 26; NaH₂PO₄ 1.25; KCl 3; CaCl₂ 2; MgSO₄ 1; D-glucose 10; pH 7.4, when supplied with 95% O₂/ 5% CO₂. The brain was then bisected and glued to a metal plate with the mid-line uppermost and horizontal. The mounted tissue was then submerged in oxygenated (95% O₂/ 5% CO₂) aCSF, before being cut into 400 μ m sagittal hippocampal slices by a Vibratome (IntraCel, Royston, Herts, UK). Such slices were then maintained in an incubation chamber containing circulating oxygenated aCSF (room temperature) on a submerged nylon mesh, for at least 1 h prior to use.

2.10) Extracellular recording from hippocampal CA1 pyramidal neurons.

The recording conditions were as we previously described (Zhang et al., 2013). The hippocampal slice was submerged in recording chamber (Scientific Systems Design Paris France), perfused ($4 - 6 \text{ ml min}^{-1}$; oxygenated 95% O_2 / 5% CO_2), with aCSF maintained at 32°C by a Digitimer PTC03 temperature controller. To induce basal field excitatory postsynaptic potentials (fEPSPs) a stimulating tungsten bipolar electrode ($\sim 100 \text{ }\mu\text{m}$ diameter) was located within the *stratum radiatum*, and a Digitimer stimulator (0.033 Hz; $100 \text{ }\mu\text{s}$ duration) used to excite the afferent Schaffer collateral-commissural pathway from the CA3 area to the CA1 region. A glass (King Precision Glass ID = $1.0 \pm 0.05 \text{ }\mu\text{m}$; OD = $1.55 \pm 0.05 \text{ }\mu\text{m}$) extracellular recording electrode was filled with aCSF ($< 5 \text{ M}\Omega$) and carefully lowered into *stratum radiatum* dendritic region of CA1 until clear fEPSPs were evident. The signal was amplified by a Warner Instruments Corp. differential amplifier with the fEPSPs simultaneously displayed on a digital storage oscilloscope (Tektronix 2201) and on a computer screen via an A to D converter (National Instruments, Paris, France; BNC-2090). The stimulus parameters, the acquisition and the analysis of fEPSPs was controlled by an LTP software suite (provided by Professor Collingridge and Dr. Anderson; Bristol University U.K.). The slope (mV ms^{-1}) and amplitude (mV) of each fEPSP was calculated on line and the stimulus adjusted to produce a response 40% of the maximum. All fEPSPs were acquired at 10 kHz and filtered at 10 Hz to 3 kHz.

To investigate the ability of S44819 to induce repetitive firing, in response to a single electrical stimulus of the Schaffer collateral pathway, fEPSPs (elicited at 0.033 Hz, 0.1 ms duration) were recorded for 10 min to establish consistent controls before the bath perfusion of S44819, or bicuculline. To induce long term potentiation (LTP) a maximal 4 pulse, or submaximal 3 pulse theta-burst (TBS) stimulation protocol was employed. The duration of each single stimulation = 0.1 ms, which was delivered in a group of 4 or 3 pulses at a frequency of 100 Hz, repeated 10 times with an interval of 200 ms between each of the 10 clusters of 4, or 3 pulses) – see (Zhang et al., 2013). and assessment of the magnitude of LTP was made by taking the mean of the fEPSP slopes for each slice between 50 – 60 min

post the TBS. Statistical analysis of LTP was performed with SPSS software employing a one-way ANOVA followed by a *post hoc* Tukey HSD multiple comparison test.

2.11) Object recognition assay in rats.

Male Sprague-Dawley (SPRD) rats bred in house were used at 290-350 g body weight (BW). All animals were housed under standard laboratory conditions (24 ± 2 °C, 40–60% relative humidity), on a 12-h light/dark cycle with light onset at 6:00 am (Initial Zeitgeber 3). All experimental protocols were approved by the Animal Care and Use Ethical Committee of Egis Pharmaceuticals PLC and complied with the Hungarian Law of Animal Care and Use (1998. XVIII). The object recognition protocol was as previously described (Gacsályi et al., 2013). Briefly, on day 0 (familiarization) the test animals were placed in the test box one by one for 2.5 min each. On the next day, animals were placed in the test box to explore two identical objects 120 min after *p.o.* administration of either nano-encapsulated S44819 consisting of S44819 3.8/Lipoïd S100 (Lipoïd GmbH, Ludwigshafen, Germany) 38.5/trehalose 57.7, w/w/w) corresponding to S44819 (0.1, 0.3 and 1mg/kg), or the vehicle (Lipoïd S100/trehalose, 40%/60%, w/w). The animal was returned to its home cage once the exploration times of both objects reached 10 sec/object within a cut-off time of 5 min (acquisition). The amount of time spent exploring the arena and the time of investigative contact with each object was recorded. Twenty-four hours later, rats were allowed to explore the familiar object and a new object in the place of the second familiar one in the same arena. The timings of the test were adjusted so that vehicle-treated animals did not discriminate between the familiar and the new object 24h after the first trial. The calculated parameter was the discrimination index: $DI = [\text{Exploration time of new object in seconds (N)} - \text{Exploration time of familiar object in seconds (F)}] / [\text{exploration time of new object in seconds (N)} + \text{Familiar object exploration time in seconds (F)}]$; $DI = [N - F] / [N + F]$. One-way ANOVA was used to analyse the effect of treatment with S44819 on object recognition

performance, followed by Dunnett's test for multiple comparisons. All analysis was by GraphPad Prism v6.0 (GraphPad Software, San Diego, USA).

2.12) Working memory in eight-arm radial maze in rats.

Male Sprague Dawley (SPRD) rats (Charles River, Germany and Egis Pharmaceuticals PLC, Hungary), (240-310 g BW) were trained and tested in an eight-arm-maze-based spatial working memory task (Gacsályi et al., 2013). Upon partial restriction of food (receiving approximately 8-10 g standard rodent food/day/animal), the rat was rewarded by bait when it entered a previously unvisited arm of the maze. Training took place once a day for three weeks. The total number of errors (TE) and the number of correct entries until the first mistaken choice (ICR) were recorded. Animals included in the study had TE values < 3 and $ICR \geq 7$. The testing of the compound was on the last day of the experiment. Scopolamine hydrobromide (0.25 mg/kg), or saline were injected s.c., immediately followed by the *i.p.* administration of either S44819 (1 and 3 mg/kg), or vehicle (0.4 % methyl cellulose w/v) 30 min before the start of the test. A single trial lasted a maximum of 5 min. The test was validated by comparing the vehicle/vehicle treated group with the group receiving scopolamine/vehicle by Mann-Whitney U-test. The effect of S44819 was analysed by non-parametric Kruskal-Wallis ANOVA, *post-hoc* comparisons were performed using the Dunn's multiple comparisons test.

3. RESULTS

3.1) S44819 is a competitive inhibitor of recombinant $\alpha 5$ -GABA_ARs.

The FLIPR assay revealed a potent inhibition by S44819 (1 nM – 10 μ M) of the GABA-induced response (a change in fluorescence considered to report cellular depolarisation) mediated by recombinant $\alpha 5\beta 2\gamma 2$ GABA_ARs expressed in HEK293 cells (Figure 1A). S44819 exhibited a preference for the $\alpha 5$ -GABA_AR subtype, requiring greater

concentrations to inhibit equivalent GABA_ARs incorporating the $\alpha 1$, or the $\alpha 3$ subunit, with little effect on GABA-evoked responses mediated by $\alpha 2$ -GABA_ARs, until the relatively high concentration of 30 μ M. (Figure 1A, Table 1). Further studies with $\alpha 5$ -GABA_ARs demonstrated that increasing concentrations of S44819 (100 nM – 10 μ M) caused a parallel shift to the right of the GABA concentration-response relationship (Figure 1B), producing linear Schild-plots with a slope not significantly different from unity (not shown), suggesting that S44819 is a competitive $\alpha 5$ -GABA_AR antagonist ($K_b = 221$ nM - geometric mean, 95% Confidence Interval [CI] 167 to 294, $n = 7$). In contrast, inhibition of $\alpha 1\beta 2\gamma 2$ and $\alpha 3\beta 2\gamma 2$ GABA_ARs by S44819 could not be classified by Schild-analysis.

Etomidate activates GABA_ARs *via* a mechanism distinct from GABA. In contrast to the potent inhibitory effects upon GABA-activated responses mediated by $\alpha 5$ -GABA_ARs, S44819 (up to 3 μ M) had no effect on the etomidate (30 μ M)-evoked response. Consistent with previous reports, gabazine was similarly ineffective in blocking such responses, whereas they were inhibited by bicuculline in a concentration-dependent manner (Thompson et al., 1999; Ueno et al., 1997) (Figure 2).

To further characterize the interaction of S44819 with the GABA binding-site, we investigated the influence of S44819 on the binding of the GABA_AR agonist 3 H-muscimol. S44819 (0.1 nM – 100 μ M) produced a concentration-dependent displacement of the specific binding of 3 H-muscimol to membrane suspensions made from HEK293 cells previously transfected with $\alpha 5$, $\beta 3$ and $\gamma 2$ subunits ($K_i = 66$ nM geometric mean, 95% CI. 61 to 72, $n = 3$), further supporting an interaction of the compound with the GABA recognition site of the receptor (Figure 3A). In contrast, S44819 (0.1 nM – 10 μ M) had no effect on the specific binding of the benzodiazepine site ligand 3 H-flumazenil to $\alpha 5$ -GABA_ARs ($\alpha 5\beta 3\gamma 2$), whereas, the benzodiazepine-site $\alpha 5$ -GABA_AR-selective NAM Ro4938581 (Knust et al., 2009), produced the expected concentration-dependent inhibition with a K_i of 13 and 12.2 nM in two independent studies (Figure 3B).

3.2) Location of the S44819 binding site on the $\alpha 5$ -GABA_AR.

The properties of the $\alpha 5$ -GABA_AR inhibitory effect of S44819 appeared to conform to a loop-F model of action (Ling et al., 2015; Mihalik et al., 2017). First, the compounds require strict regiochemistry of the oxazolone moiety to interact with amino acid residues previously reported to interact with GABA (Ling et al., 2015; Mihalik et al., 2017). A further facet of the loop-F model is that the pharmacological properties of this class of compounds are determined by the variable, N-terminal segment of loop-F of the α -subunit (Mihalik et al., 2017). However, the impact of loop-F alterations on the inhibitory effect varied both with the compound and the α -subunit tested (Mihalik et al., 2017). For S44819, the potent inhibitory effect on the GABA-induced change in fluorescence mediated by wild type (WT) $\alpha 5$ -GABA_ARs was greatly reduced for equivalent receptors incorporating a mutant $\alpha 5$ subunit ($\alpha 5$ -LF $\alpha 2$) engineered to express the $\alpha 2$ T---TYNASD---P loop-F sequence, which replaced the native $\alpha 5$ V---NGSTK---E loop-F sequence (Figure 4). These observations were corroborated by complementary whole-cell voltage-clamp experiments, performed using HEK 293 cells ($V_h = -60$ mV), previously transfected with recombinant GABA_AR subunits. Here, a relatively low concentration of S44819 (30 nM) decreased the GABA-evoked current (100 μ M GABA transiently applied by pressure application once *per* 30 s for 50 ms from a local pipette) mediated by $\alpha 5$ -GABA_ARs to $32 \pm 4\%$ of control; $n = 3$; Figure 5). In contrast, S44819 (30 nM) had no effect on equivalent $\alpha 2$ -GABA_ARs ($92 \pm 8\%$ of control; $n = 3$, Figure 5). The potent and selective inhibition by S44819 of GABA-evoked currents mediated by $\alpha 5$ -GABA_ARs was completely prevented for GABA-evoked responses mediated by the mutant ($\alpha 5$ -LF $\alpha 2$) subunit containing receptor ($100 \pm 4\%$ of control; $n = 3$), in this regard mimicking the lack of effect of this compound on equivalent $\alpha 2$ -GABA_ARs ($92 \pm 8\%$ of control; $n = 3$) under these conditions (Figure 5).

3.3) S44819 selectively inhibits extrasynaptic $\alpha 5$ -GABA_ARs of mouse CA1 pyramidal neurons.

Given the selectivity of S44819 for recombinant $\alpha 5$ -GABA_ARs we investigated whether a similar specificity was evident for neuronal receptors. We previously demonstrated that mouse CA1 pyramidal neurons exhibit a tonic current mediated by $\alpha 5$ -GABA_ARs (Caraiscos et al., 2004). Here, we used the whole-cell voltage-clamp technique to record the tonic and phasic currents (*i.e.* the mIPSCs) mediated by extrasynaptic $\alpha 5$ -GABA_ARs and by synaptic GABA_ARs respectively. Using a hippocampal slice preparation recordings were made from CA1 pyramidal neurons ($V_h = -60$ mV), pre-incubated in the GABA-transaminase inhibitor vigabatrin (50 μ M) for 2 - 4 h to raise extracellular levels of GABA and thereby enhance the tonic current mediated by $\alpha 5$ -GABA_ARs (Caraiscos et al., 2004). Under these conditions, the bath perfusion of S44819 (10 μ M) induced an outward current (80 ± 20 pA; $n = 3$) - Figure 6A,C. Note the subsequent perfusion of bicuculline (30 μ M) to these neurons only produced a relatively small further outward current of 19 ± 5 pA ($n = 3$), suggesting S44819 at this concentration in the slice to be a nearly full $\alpha 5$ -GABA_AR inhibitor (Figures 6A, C). We deliberately used a relatively large concentration of S44819 to investigate the selectivity of the drug for extrasynaptic vs synaptic GABA_ARs in CA1 neurons. Inspection of the recordings revealed the mIPSCs to remain clearly evident in the presence of S44819 (10 μ M), whereas these phasic inhibitory events were abolished by the subsequent addition of bicuculline (30 μ M) – see Figure 6A. These observations suggest that in contrast to bicuculline, S44819 selectively inhibits the extrasynaptic $\alpha 5$ -GABA_ARs, with little, or no effect on their synaptic counterparts. In confirmation of this selectivity, in paired recordings S44819 (10 μ M) had no significant effect on the mIPSC amplitude (control = -72 ± 4 pA; S44819 = -64 ± 2 pA; paired t test $p = 0.095$; $n = 5$) – Figure 6B, D.

3.4) S44819 had no effect on the extrasynaptic $\alpha 4\beta 2\delta$ GABA_ARs, or synaptic $\alpha 1\beta 2\gamma 2$ GABA_ARs of mouse thalamic ventrobasal neurons.

We next explored whether S44819 had any effect on a tonic current known to be mediated by non- $\alpha 5$ -GABA_A extrasynaptic receptors. Our previous studies revealed the

tonic current of ventrobasal (VB) thalamic neurons to be mediated by GABA_ARs composed of $\alpha 4$, $\beta 2$ and δ subunits (Belelli et al., 2005; Brown et al., 2015; Herd et al., 2013; Peden et al., 2008). In contrast to the CA1 neurons, S44819 (10 μ M) had no effect on the holding current ($V_h = -60$ mV) of VB neurons, whereas the subsequent addition of bicuculline (30 μ M) produced the expected outward current ($n = 4$; Figure 7A, C). By postnatal day 20 we have shown the phasic inhibitory currents (*i.e* the mIPSCs) of VB neurons to be mediated by synaptic GABA_ARs composed of $\alpha 1$, $\beta 2$ and $\gamma 2$ subunits (Belelli et al., 2005; Peden et al., 2008). In common with the CA1 neurons, in paired recordings S44819 (10 μ M) had no effect on the mIPSC amplitude (control = -74 ± 4 pA; S44819 = -73 ± 4 pA; $n = 4$; $p = 0.855$, paired t-test), of P20 VB neurons (Figures 7 B, D).

3.5) The $\alpha 5$ -GABA_AR antagonist S44819 does not produce repetitive firing, but does enhance hippocampal CA1 LTP.

Non subtype-selective GABA_AR antagonists such as picrotoxin and bicuculline are known to be pro-convulsant. Having demonstrated that S44819 selectively blocked GABA-ergic tonic, but not phasic inhibition of CA1 neurons we recorded field excitatory postsynaptic potentials (fEPSPs) from mouse CA1 neurons to compare with bicuculline, the potential for S44819 to produce repetitive firing. Submaximal (40 %) control fEPSPs (elicited at 0.033 Hz) were recorded for 10 min. to establish consistent controls. In all cases ($n = 3$), the bath application of bicuculline (10 μ M) caused clear repetitive firing of the fEPSP in response to a single electrical stimulation of the Schaffer collateral pathway ($n = 3$; Figure 8Aiii). By contrast, S44819 (10 μ M) was inert in this respect ($n = 3$; Figure 8Aii, suggesting that S44819 is devoid of pro-convulsant activity. In support, in mice S44819 was not pro-convulsant, nor did it reduce the threshold for pentylenetetrazol to induce convulsions (Gacsalyi et al., 2017).

The synaptic plasticity underlying hippocampal LTP is considered a putative electrophysiological correlate of the processes underlying certain forms of learning and memory (Neves et al., 2008). Previous studies have demonstrated $\alpha 5$ -GABA_AR NAMs to

enhance hippocampal CA1 neuron LTP (Atack, 2011). To investigate whether S44819 similarly influenced this form of synaptic plasticity we recorded fEPSPs from mouse CA1 neurons. Again, submaximal (40 %) control fEPSPs (0.033 Hz) were recorded for 10 min. We previously reported that a 4-pulse theta burst stimulus (4-TBS) protocol induced maximal LTP, whereas a 3-pulse protocol (3-TBS) was submaximal (Zhang et al., 2013). In agreement, delivery of a 4-TBS induced a robust form of LTP, determined 50 min post the TBS (fEPSP slope = $195 \pm 10\%$ of control, $n = 9$), whereas that produced by a 3-TBS was significantly less effective (fEPSP slope = $124 \pm 6\%$ of control; $n = 6$) (Figures 8B, C). S44819 (0.3 μM) greatly increased the fEPSP slope induced by a 3-TBS at 60 min after the TBS, ($191 \pm 14\%$ of control; $n = 4$) ----Figures 8B, C. In conclusion, for hippocampal CA1 neurons S44819 (10 μM) did not induce repetitive firing, but enhanced this form of synaptic plasticity (LTP) at a much lower concentration (300 nM).

3.6) Effects of S44819 on learning and memory.

In male rats, S44819 (0.1 -1 mg/kg *p.o.*) given 120 min prior to the acquisition trial in the object recognition assay increased the time spent investigating the novel object over the familiar object when assessed 24 h later (Figure 9A). Other dosage regimes of the drug were not tested. In the eight-arm radial maze, S44819 (1 and 3 mg/kg *i.p.*) was given simultaneously with scopolamine 30 min prior to the start of the test trial. S44819 significantly diminished the marked increase in total errors induced by scopolamine (Figure 9B). In both paradigms the effects of S44819 appeared dose-dependent.

4. DISCUSSION

This study describes the properties of S44819, a novel, potent, selective and competitive inhibitor of $\alpha 5$ -GABA_ARs. Drugs previously reported to selectively impair the function of $\alpha 5$ -GABA_ARs do so by binding to the benzodiazepine site, located between the α and the γ subunit, to produce a negative allosteric effect and are classed as NAMs (Atack, 2011; Olsen and Sieghart, 2008; Rudolph and Möhler, 2014). Drugs such as L655,708 and

Ro4938581 exhibit binding selectivity, whereas others have similar binding affinities for most GABA_AR isoforms, but are functionally selective NAMs *e.g.* α 5IA (Atack, 2011). By contrast, evidence is presented here that S44819 competes with the neurotransmitter GABA for a binding site on the α 5-GABA_AR. Furthermore, the selectivity for this receptor isoform is governed by key amino acids residues in loop F of the α 5 subunit. Note these amino acids are distinct from those α -subunit residues important for benzodiazepine binding. However, in common with known α 5-GABA_AR NAMs, S44819 exhibited pro-cognitive activity in rodent behavioural studies.

S44819 produced a potent inhibition of both the GABA-induced response (reported by a membrane potential-sensitive dye) and the GABA-evoked current mediated by recombinant α 5-GABA_ARs expressed in HEK293 cells. A Schild-plot analysis of the effect of S44819 on the GABA concentration-response relationship suggested that S44819 is a competitive inhibitor of α 5-GABA_ARs. In agreement, S44819 displaced the specific binding of the GABA_AR agonist ^3H -muscimol to recombinant α 5-GABA_ARs, but in contrast to known selective α 5-GABA_AR NAMs, had no effect on the specific binding of the benzodiazepine ^3H -flumazenil. Computer modelling and site-directed mutagenesis implicated a role for the variable, NH₂-terminal segment of loop-F of the α subunit in determining the pharmacological profile of structurally-related novel tricyclic compounds (Mihalik et al., 2017). However, as inhibitory potency varied both with the compound tested and with the GABA_AR isoform under study, it was important to now characterize S44819 in this respect. Significantly, in both the membrane potential dye and in the voltage-clamp assays the inhibitory potency of S44819 was markedly reduced upon mutation of the NH₂-terminal segment of loop-F of the α 5 subunit. Collectively, the data summarized above indicate that in common with other compounds containing an oxazolo-2,3 benzodiazepine scaffold (Mihalik et al., 2017), S44819 docks in the L-shaped GABA binding pocket (Bergmann et al., 2013) and an important element of the GABA_AR isoform selectivity of the drug is an interaction with the variable segment of Loop-F of the α -subunit.

To investigate in mouse hippocampal slices the native GABA_AR-isoform selectivity of S44819 we deliberately used a relatively high concentration (10 μ M) of the inhibitor. This concentration of S44819 suppressed the tonic current mediated by extrasynaptic α 5-GABA_ARs (Caraiscos et al., 2004), but had no effect on the amplitude of mIPSCs mediated by synaptic GABA_ARs, whereas bicuculline blocked both phasic and tonic inhibition. Although most α 5-subunits are expressed extrasynaptically, EM studies reveal them to be present within certain synapses of hippocampal pyramidal cells (Serwanski et al., 2006), with their location governed by the anchoring protein radixin (Hausrat et al., 2015). Indeed, a proportion of inhibitory phasic events with relatively slow kinetics, which in some neurons are sensitive to L655,708, an α 5-GABA_AR NAM, have been reported (Capogna and Pearce, 2011; Prenosil et al., 2006; Salesse et al., 2011; Zarnowska et al., 2009). However, such events are primarily evident under conditions, which permit multi-vesicular GABA release (electrically evoked [e] IPSCs, or spontaneous [s] IPSCs). Therefore, the lack of effect of S44819 on mIPSCs (recorded in tetrodotoxin to block presynaptic action potentials) reported here is not unexpected. For thalamic VB neurons S44819 (10 μ M) had no effect on the mIPSC amplitude mediated by synaptic GABA_ARs (α 1 β 2 γ 2), or on the tonic current due to extrasynaptic α 4 β 2 δ receptors (Belelli et al., 2005; Peden et al., 2008), observations that further emphasise the α 5-GABA_AR selectivity of the drug.

Therefore, in brain slice experiments S44819 is highly selective for extrasynaptic α 5-GABA_ARs. There are several factors that may contribute to this selectivity: 1) The GABA_AR subunit selectivity evident from our studies on recombinant GABA_ARs; 2) Given the competitive nature of the antagonism, the marked difference between synaptic (\geq 10 μ M (Jones and Westbrook, 1995; Karayannis et al., 2010; Maconochie et al., 1994)), and extrasynaptic concentrations of GABA (\leq 2 μ M, (Lerma et al., 1986; Wlodarczyk et al., 2013)); 3). A possible greater affinity of GABA for thalamic δ -GABA_ARs *c.f.* CA1 α 5 β γ 2 GABA_ARs, coupled with an element of subunit selectivity, resulting in a clear differential effect of S44819 to block the hippocampal tonic current. However, note that in contrast to thalamus, the hippocampal experiments were conducted after treatment with vigabatrin to raise local

GABA concentrations. 4) The properties of native synaptic and extrasynaptic GABA_ARs may not be faithfully replicated by their recombinant counterparts expressed in cell lines.

The brain slice studies were performed on “quiescent”, non-stimulated slices. However, we previously showed that physiological frequencies of presynaptic stimulation in the thalamus produced synaptic spill-over of GABA causing a large increase in the tonic current and a considerable prolongation of phasic inhibition (Herd et al., 2013). Given that S44819 is a competitive antagonist, equivalent studies for the hippocampus e.g. using TBS frequencies of stimulation would be of interest. Furthermore, a recent report identified a large tonic current mediated by $\alpha 5$ -GABA_ARs in a sub-population of central amygdala neurons that was insensitive to gabazine, but was inhibited by picrotoxin and by L-655,708, an $\alpha 5$ -GABA_AR selective NAM, suggesting that these receptors spontaneously conduct anions (Botta et al., 2015). Clearly, it would be of future interest to determine the effect of S44819 on such neurons to establish whether it differentiates between these extrasynaptic $\alpha 5$ -GABA_ARs of hippocampus and amygdala.

GABA_AR antagonists such as bicuculline do not exhibit GABA_AR isoform selectivity and consequently are pro-convulsant. As expected, in the hippocampus bicuculline inhibited both synaptic and extrasynaptic GABA_ARs and consequently produced repetitive firing. However, in contrast, relatively high concentrations of S44819 (10 μ M) did not inhibit synaptic GABA_ARs and did not produce repetitive firing. Indeed, in mice behaviourally-active doses of S44819 are not pro-convulsant, nor do they reduce the dose of pentylenetetrazol required to induce convulsions (Gacsályi et al., 2017). Selective $\alpha 5$ -GABA_AR NAMs facilitate hippocampal LTP (Atack, 2011). Similarly, a low concentration (300 nM) of S44819 enhanced sub-maximal LTP of hippocampal CA1 neurons. Such effects on synaptic plasticity may contribute to the pro-cognitive actions of this compound. The effect on LTP, coupled with a lack of pro-convulsant activity *c.f.* bicuculline, further suggests that in hippocampal CA1 neurons S44819 acts as a selective inhibitor of $\alpha 5$ -GABA_ARs. This selective effect on hippocampal synaptic plasticity warranted assessment of the pro-cognitive potential of S44819.

A prominent involvement of $\alpha 5$ -GABA_ARs in object recognition memory is well established. Administration of an $\alpha 5$ -GABA_AR positive allosteric modulator reduced the preference for the novel object, whereas $\alpha 5$ -GABA_AR inhibitors enhanced it (Ling et al., 2015; Milic et al., 2013; Redrobe et al., 2012). In the version of the test used here, in rats S44819 administered before the start of the acquisition trial effectively enhanced novel object preference measured 24 h later. The brain circuits that underlie novel object recognition in the rat are primarily within the perirhinal and entorhinal cortices. (Barker and Warburton, 2011; Brown and Banks, 2015; Keene et al., 2016; Kinnavane et al., 2014). The role of tonic inhibition in these brain regions has not been studied in detail. However, mRNA expression studies in models of temporal epilepsy indicate that the molecular components of tonic inhibition are expressed in these cortical regions and show marked changes upon exposure to convulsive agents, suggestive of functional relevance (Drexel et al., 2013).

The radial maze paradigm used here tests spatial working memory in an appetitive task. This test prominently involves the hippocampus, where the relevance of $\alpha 5$ -GABA_AR mediated tonic inhibition for the activation of principal neurons is well documented (Bonin et al., 2007; Caraiscos et al., 2004). Accordingly, S44819 was effective in ameliorating the profound deficit of working memory evoked by an acute injection of scopolamine. A likely mechanism underlying this effect of S44819 is the lowering of the firing threshold of CA1 principal neurons, thereby enabling cholinergic triggering of hippocampal network oscillations (Cobb and Davies, 2005; Fisahn et al., 1998; Huerta and Lisman, 1995) in the face of inhibition by scopolamine. We have now reported a further characterisation of the behavioural actions of S44819 in preclinical models (Gacsályi et al., 2017). Consistent with these behavioural investigations, pharmacokinetic studies in rodents reveal S44819 to achieve active brain concentrations (brain:plasma ratio of 1:2 for male mice and 1:4 for male rats), with an appropriate time course (Gacsályi et al., 2017). Importantly, in clinical studies the EEG signal evoked by transcranial magnetic stimulation of the human neocortex indicated an enhancement of neuronal excitability after treatment with S44819 (Darmani et

al., 2016). Thus, in humans S44819 similarly reaches the CNS in concentrations sufficient to elicit a biological response.

In conclusion, S44819 is a selective, competitive, inhibitor of extra-synaptic $\alpha 5$ -GABA_ARs and provides a new mechanistic tool to elucidate the role of $\alpha 5$ -GABA_ARs in neuronal signaling and behavior. Although acting by a distinct mechanism to known $\alpha 5$ -GABA_AR NAMs, in common with such drugs S44819 exhibits pro-cognitive activity in rodent behavioral studies. Given the potential for $\alpha 5$ -GABA_AR inhibitors to treat cognitive disorders (Atack, 2011) and to improve functional recovery following a stroke (Clarkson et al., 2010; Lake et al., 2015), these studies reveal an alternative site on the GABA_AR to target for therapeutic exploitation. Indeed, S44819 is currently commencing Phase 2 clinical trials.

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LEGENDS TO THE FIGURES

Figure 1: S44819 selectively inhibits $\alpha 5$ -GABA_ARs. The effect of S44819 on the GABA-induced response mediated by GABA_AR isoforms, stably expressed in HEK293 cells, was investigated by use of the FLIPR dye assay. **A.)** The effect of S44819 on the response to GABA (1.6 μ M, EC₃₀₋₅₀) was recorded from HEK cells stably expressing, $\alpha 1$ (●), $\alpha 2$ (■), $\alpha 3$ (▲) or $\alpha 5$ (▼) - GABA_AR subunits together with $\beta 2$ and $\gamma 2$ subunits. Data were expressed as a percentage of the control GABA response and represent the results of 3, 2, 4 and 3 independent experiments for $\alpha 1$ -, $\alpha 2$ -, $\alpha 3$ -, $\alpha 5$ -GABA_ARs, respectively. Data represent the mean \pm S.E.M. $n = 3 - 12$ per point. **B.)** GABA concentration-response curves obtained from cells expressing $\alpha 5\beta 2\gamma 2$ GABA_ARs in the presence of vehicle (●), 100 (■), 300 (▲), 1000 (▼), 3000 (◆) and 10000 (□) nM S44819. Data are mean \pm S.E.M, $n = 3 - 21$ /group, collected from seven independent studies. Values for GABA EC₅₀ were estimated by non-linear regression with a least squares fit in Graphpad Prism v.6.0f.

Figure 2: S44819 does not inhibit the activation of $\alpha 5$ -GABA_ARs by etomidate. The FLIPR dye assay was used to investigate the putative inhibitory effect of S44819 (◆), gabazine (▲) and bicuculline (●) on the etomidate (30 μ M) - evoked response elicited from HEK293 cells stably expressing $\alpha 5\beta 2\gamma 2$ GABA_ARs. Whereas bicuculline produced a concentration-dependent inhibition of the etomidate-evoked response, both S44819 and gabazine were relatively ineffective in this respect. Data were expressed as a percentage of the etomidate (30 μ M) – evoked control response (□). Data give the mean \pm S.E.M. and are representative of two independent experiments, $n = 3$ /point except for the control stimulus where $n = 10$.

Figure 3: S44819 displaces the specific binding of 3 H-muscimol, but not 3 H-flumazenil to $\alpha 5$ -GABA_ARs. Radio-ligand binding studies were performed with membranes prepared

from HEK293T cells transiently expressing $\alpha 5\beta 3\gamma 2$ GABA_ARs. **A.)** Displacement of ^3H -muscimol by S44819. Data are the mean \pm S.E.M. $n = 6 - 9/\text{point}$ and are representative of two independent experiments. **B)** S44819 (\blacktriangle) does not displace ^3H -flumazenil. Note the potency of the reference compound Ro4938581 (\blacksquare) - (Knust et al., 2009). The data represent the means \pm S.E.M., $n = 4/\text{point}$ and are representative of two independent experiments.

Figure 4: The inhibitory effect of S44819 is governed by α subunit F loop residues.

FLIPR dye studies were performed in HEK293 cells stably expressing GABA_AR $\beta 2$ and $\gamma 2$ subunits and transiently transfected with the WT $\alpha 5$ subunit (\bullet) or an $\alpha 5$ subunit engineered to incorporate the $\alpha 2$ subunit F loop sequence (\blacktriangle), that replaces the native $\alpha 5$ F-loop sequence ($\alpha 5\text{-LF}\alpha 2$) - (Mihalik et al., 2017). Although the F loop mutation had little effect on the GABA concentration response curves (not shown), it greatly reduced the inhibitory effect of S44819. The inset shows the amino acid sequence modifications to the $\alpha 5$ subunit F loop (shaded in grey). Data give the mean \pm S.E.M, $n = 3/\text{group}$, and are representative of two independent studies.

Figure 5: S44819 selectively inhibits GABA-evoked currents mediated by $\alpha 5$ -GABA_ARs, a specificity governed by $\alpha 5$ subunit loop-F residues.

The bath application of S44819 (30 nM) potently inhibited GABA-evoked whole-cell currents mediated by $\alpha 5\beta 2\gamma 2$ GABA_ARs transiently expressed in HEK-293 cells (\bullet). By contrast, this concentration of S44819 had no effect on equivalent GABA-evoked responses mediated by $\alpha 2\beta 2\gamma 2$ receptors (\blacksquare), or on recombinant receptors engineered to express a mutant $\alpha 5$ subunit ($\alpha 5\text{-LF}\alpha 2$) $\beta 2\gamma 2$ (\blacktriangledown), where key amino acid residues in the loop F region have been exchanged to the equivalent loop F residues of the GABA_AR $\alpha 2$ subunit (see Figure 4). The graph illustrates the time course of the effect of S44819 on GABA-evoked currents, with time = 0 indicating the time from switching from control saline, to a solution containing S44819 (30

nM). Note there is an ~ 60 s perfusion lag-time required before the drug enters the recording chamber. For each experiment, at least 3 control GABA (100 μ M; transiently applied by pressure for 50 ms from a local pipette once *per min.*) - evoked currents were recorded, with their amplitudes not varying by more than 10%, before S44819 (30 nM) was bath applied. The current amplitudes were normalized with respect to the first control amplitude. The mean normalized \pm S.E.M. data were derived from 3 - 5 cells for each condition. The inset depicts representative GABA-evoked currents mediated by $\alpha 5\beta 2\gamma 2$ GABA_ARs prior to and after the application of S44819 (30 nM).

Figure 6: Selective inhibition of the GABA-mediated tonic current of mouse CA1 pyramidal neurons by S44819. A.) A representative trace of a whole-cell voltage-clamp recording from a mouse CA1 pyramidal neuron (previously incubated in vigabatrin- see Methods), demonstrating that S44819 (10 μ M) inhibits the majority of the tonic current in these neurons, as evidenced by the modest additional effect of bicuculline (30 μ M) when co-applied with S44819 (10 μ M). The broken horizontal lines illustrate the mean holding current in control, in the presence of S44819 and in the presence of S44819 + bicuculline. Inspection of the trace reveals S44819 to produce a clear outward current, but that the mIPSCs are still evident, whereas they are abolished by the subsequent addition of bicuculline. **B.)** In a paired recording a more detailed analysis of phasic inhibition revealed S44819 (10 μ M) to have no effect on the amplitude of the representative ensemble averages of mIPSCs recorded before (black trace) and after (grey trace) the bath application of S44819 (10 μ M). **C. D.)** Bar graphs (mean \pm S.E.M.) summarizing the effects of S44819 (10 μ M) and of bicuculline (30 μ M) + S44819 (10 μ M) upon **C)** the tonic current (n = 3 neurons) and **D)** of S44819 (10 μ M) upon the mIPSC peak amplitude (n = 5 neurons).

Figure 7: S44819 has no effect on the tonic, or the phasic inhibitory currents (mIPSCs) of thalamocortical neurons A.) A representative trace of a whole-cell voltage-clamp recording from a ventrobasal (VB) neuron demonstrating that S44819 (10 μ M) does

not produce an outward current *i.e.* it does not inhibit the resident tonic current in these neurons. The subsequent application of bicuculline (30 μ M) produced an outward current and abolished the mIPSCs. The broken horizontal lines illustrate the mean holding current before and during S44819 and after S44819 + bicuculline. **B.)** S44819 (10 μ M) had no effect on the properties of synaptic GABA_ARs, as illustrated by the representative ensemble averages of mIPSCs recorded before (black trace) and after (grey trace) the bath application of the drug. **C, D).** Bar graphs (mean \pm S.E.M.) summarizing the effects of S44819 (10 μ M) and of bicuculline (30 μ M) on **C)** the tonic current ($n = 4$) and **D)** of S44819 (10 μ M) on the mIPSC the peak amplitude ($n = 4$).

Figure 8: S44819 does not produce repetitive firing, but facilitates hippocampal CA1 long-term potentiation (LTP). **A.)** Illustrated are individual field excitatory postsynaptic potential (fEPSPs) recorded from the dendritic field of mouse hippocampal CA1 neurons. The fEPSPs are elicited by electrical stimulation of the Schaffer Collateral pathway (once every 30 sec.) and are from a single representative experiment of 3 separate experiments. **Ai)** A control fEPSP, **Aii)** a fEPSP in the presence of S44819 (10 μ M) and **Aiii)** a fEPSP in the presence of bicuculline (10 μ M). Note this relatively high concentration of S44819 had no effect on the fEPSP, whereas bicuculline caused clear repetitive firing in response to a single electrical stimulus. **B.)** For the LTP experiments the slope of the fEPSP is plotted as a function of time before and after delivery of a 3- or 4-pulse theta burst stimulation (-TBS), delivered at $t = 10$ min. The 3-TBS is designed to induce a submaximal form of LTP (\bullet), *c.f.* that produced by the 4-TBS paradigm (\bullet). S44819 (300 nM) clearly enhanced the submaximal LTP induced by the 3-TBS (\bullet). Note S44819 was present for the 10 min prior to delivery of the 3-TBS. Each time point (one every 30 sec.) represents the mean \pm s.e.m. of 4 – 9 independent experiments. **C.)** The histogram compares the magnitude of LTP (%age of the control fEPSP slope determined at 60 min. *i.e.* 50 min post the TBS) for control 4-TBS (black), control 3-TBS (dark grey) and 3-TBS + 300 nM S44819 (light grey). Each bar represents the mean \pm S.E.M. of 4 - 9 independent experiments. A one-way

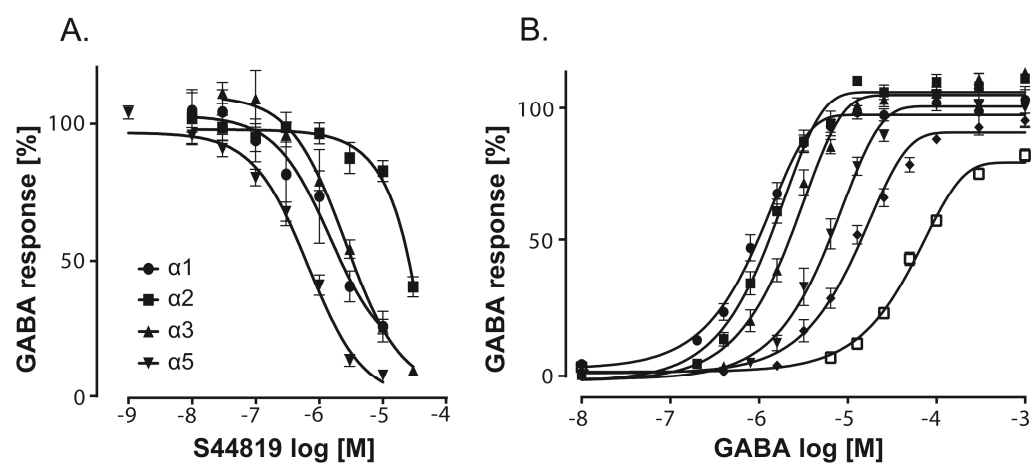
ANOVA showed significant differences between treatments ($p = 0.0002$). A *post hoc* Tukey HSD multiple comparison test revealed a significant difference between the magnitude of LTP produced by a) the control 3-TBS and the control 4-TBS $p = 0.001^{**}$, b) between the control 3-TBS and the 3-TBS + S44819 $p = 0.002^{*}$ with c) no significant difference between the 4-TBS and the 3-TBS + S44819 ($p = 0.961$).

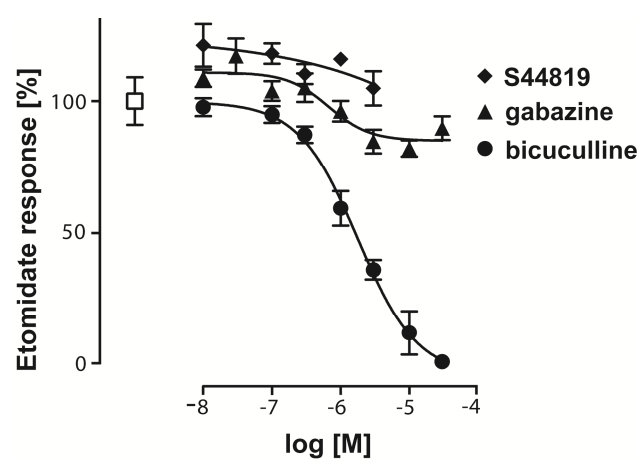
Figure 9: The pro-cognitive efficacy of S44819. A.) A histogram illustrating the performance of male rats in the object recognition test assessed 24 h after the first exposure (acquisition) to two identical objects. S44819 (0.1, 0.3 and 1mg/kg nano F3), or vehicle (lipoid S100/trehalose, 40%/60%, w/w) were given *p.o.* 120 min before the acquisition trial. Data are expressed as the discrimination index $[DI] = (N-F/N+F)$, where N is the time spent investigating the new object and F is the time spent investigating the familiar object). 1-way ANOVA $F(3,34) = 7.666$, $p = 0.0005$, followed by Dunnett's test for multiple comparisons, $*p = 0.0352$, $***p = 0.0001$. **B.)** S44819 ameliorates the impairment of spatial working memory caused by scopolamine. Scopolamine hydrobromide (0.25 mg/kg), or saline were injected *s.c.*, immediately followed by the *i.p.* administration of either S44819 (1 and 3 mg/kg) or vehicle only (0.4 % methyl cellulose w/v) 30 min before the start of the test. The total error counts are shown, mean \pm S.E.M., $n = 9 - 10$ rats/group $###p = 0.0001$ compared to the group (veh/veh) not receiving scopolamine, Man-Whitney U-test. To evaluate the effect of S44819 on the scopolamine-impaired performance *c.f.* the vehicle treated group that received scopolamine, the Kruskal-Wallis test $H(3,30) = 26.722$, $p = 0$, followed by Dunn's multiple comparisons test, $p = 0.0332$, $***p = 0.0001$ was used.

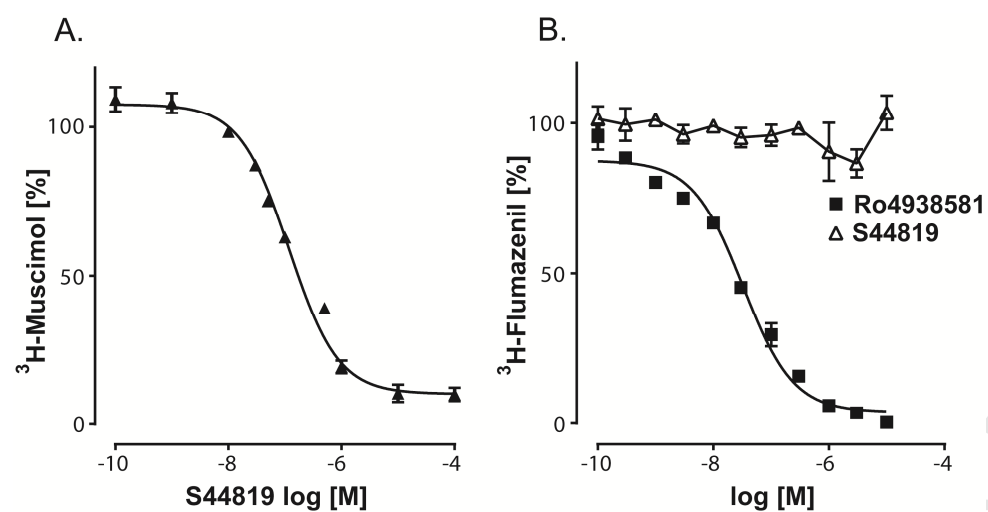
Table 1: The GABA_AR subtype selectivity of S44819. The subtype selectivity of S44819 was determined by use of the FLIPR assay to determine the inhibitory effect of S44819 on the GABA (1.6 μ M) - induced depolarisation of HEK293 cells stably expressing recombinant GABA_ARs.

Receptor isoform	Geometric mean of IC ₅₀ (nM)	95% confidence interval [nM]	N
$\alpha 1\beta 2\gamma 2$	2378	1319 to 4285	3
$\alpha 2\beta 2\gamma 2$	N.D.	-	2
$\alpha 3\beta 2\gamma 2$	3695	1752 to 7793	4
$\alpha 5\beta 2\gamma 2$	585	386 to 886	3

N.D. = could not be determined

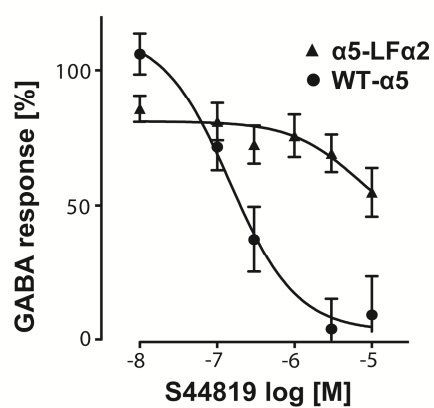


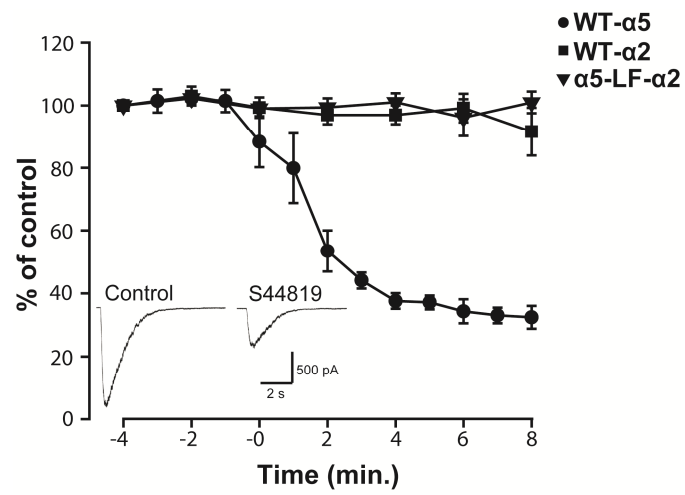


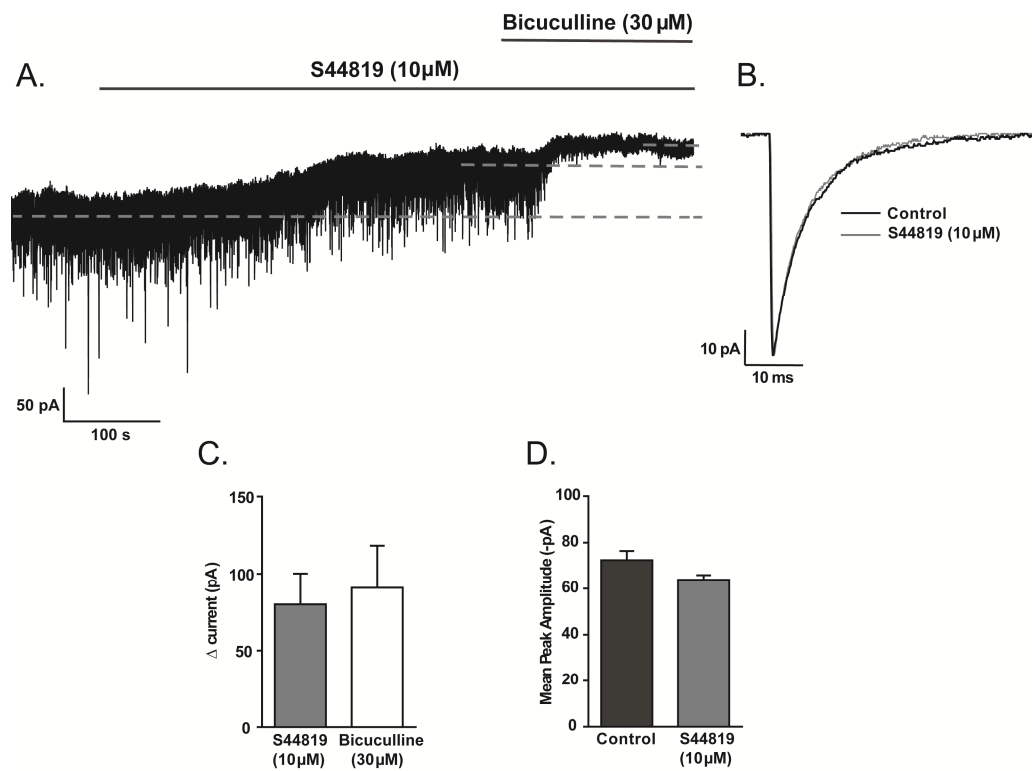


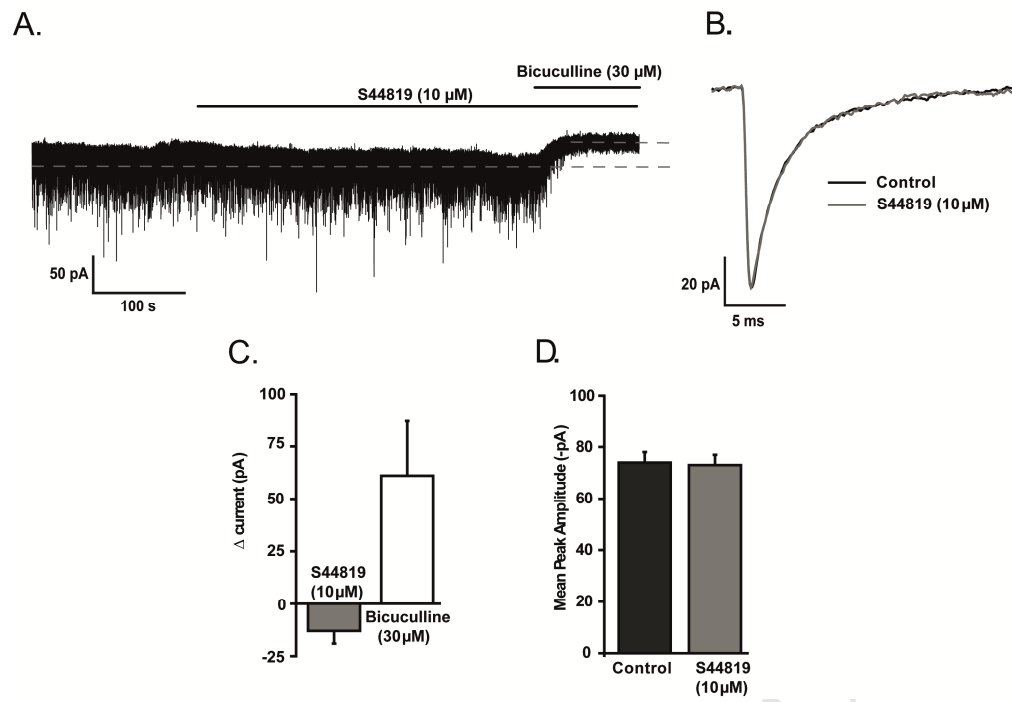
WT- $\alpha 5$: V V Y V W T N G S T K S V V V A E D G

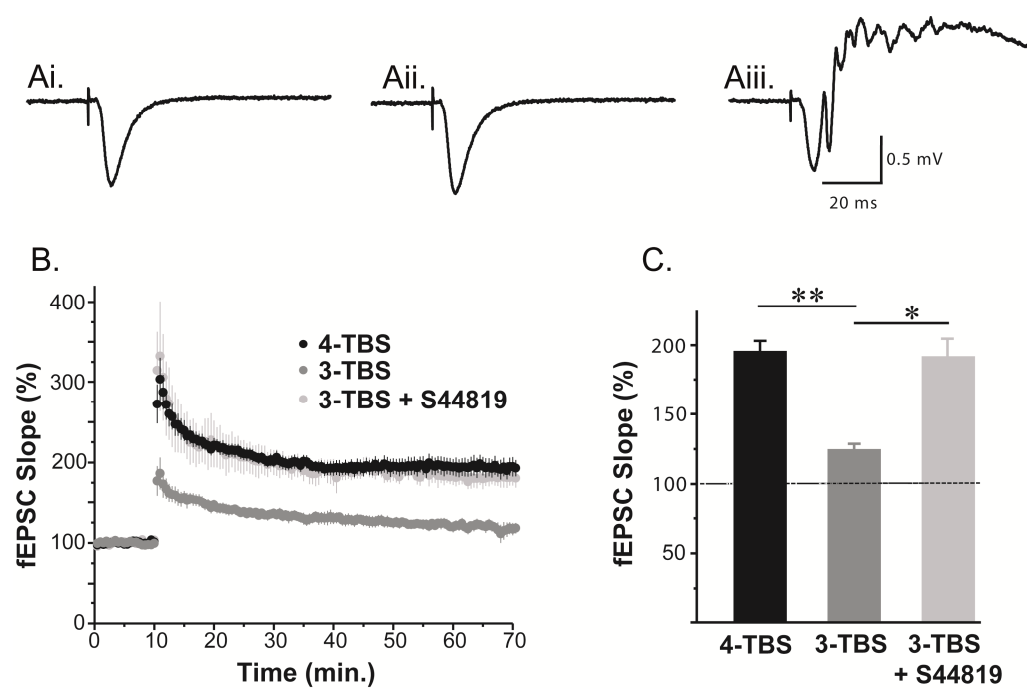
$\alpha 5$ -LF- $\alpha 2$: V **T** Y V W T **Y N A S D** S V V V A **P** D G

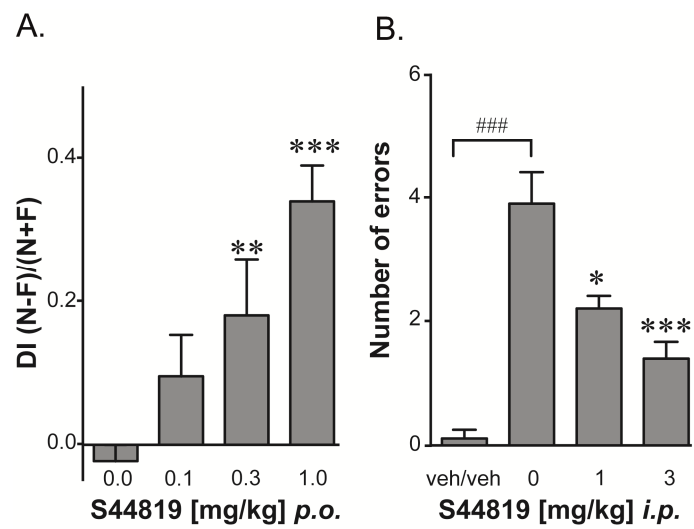












All current, selective, inhibitors of $\alpha 5$ -GABA_ARs bind to the “benzodiazepine site”.

S44819 acts *via* a unique site on the $\alpha 5$ -GABA_AR to competitively inhibit GABA binding.

S44819 inhibits mouse hippocampal tonic, but not phasic inhibition & facilitates LTP.

In rats S44819 enhanced novel object recognition & spatial working memory.

This novel $\alpha 5$ -GABA_AR site offers a new target to treat cognitive disorders & stroke.

S44819 has successfully completed a phase1 clinical trial.